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Quantitative Analysis of the Growth of *Salmonella* Stanley during Alfalfa Sprouting and Evaluation of *Enterobacter aerogenes* as Its Surrogate

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ABSTRACT

Raw seed sprouts have been implicated in several food poisoning outbreaks in the last 10 years. Few studies have included investigations of factors influencing the effectiveness of testing spent irrigation water, and in no studies to date has a nonpathogenic surrogate been identified as suitable for large-scale irrigation water testing trials. Alfalfa seeds were inoculated with *Salmonella* Stanley or its presumptive surrogate (nalidixic acid–resistant *Enterobacter aerogenes*) at three concentrations (~3, ~30, and ~300 CFU/g) and were then transferred into either flasks or a bench top–scale sprouting chamber. Microbial concentrations were determined in seeds, sprouts, and irrigation water at various times during a 4-day sprouting process. Data were fit to logistic regression models, and growth rates and maximum concentrations were compared using the generalized linear model procedure of SAS. No significant differences in growth rates were observed among samples taken from flasks or the chamber. Microbial concentrations in irrigation water were not significantly different from concentrations in sprout samples obtained at the same time. *E. aerogenes* concentrations were similar to those of *Salmonella* Stanley at corresponding time points for all three inoculum concentrations. Growth rates were also constant regardless of inoculum concentration or strain, except that lower inoculum concentrations resulted in lower final concentrations proportional to their initial concentrations. This research demonstrated that a nonpathogenic easy-to-isolate surrogate (nalidixic acid–resistant *E. aerogenes*) provides results similar to those obtained with *Salmonella* Stanley, supporting the use of this surrogate in future large-scale experiments.

During the past decade, the consumption of raw sprouts has become the focus of increasing concern because of numerous foodborne disease outbreaks and the recall of contaminated seed lots *(23, 27, 29, 38)*.

It is commonly accepted by those in the industry that contaminated sprouts result from seeds that are contaminated prior to the sprouting process *(27)*. Sprouted seeds are unique as a food raw material in that most of the seed produced in a given year is used for agricultural purposes (i.e., it is planted in the ground), and only a small proportion is sold to sprout producers for use as human food. Cross-contamination also can occur anytime during seed storage, transportation, or processing, which each may involve combining different lots of seeds from various origins *(27)*. Although control of microbial contamination prior to the arrival of seed at a sprout producer's facility is desirable, it may be difficult to achieve. Thus, interventions or preventative measures have been focused on the sprout production facility immediately prior to the sprouting process.

Some research has indicated that simply storing contaminated seeds will result in a decline in pathogen concentration. When seeds inoculated with *Salmonella* at 2 to 3 log CFU/g were stored at 8°C, there was a 1-log decrease in the organism after 8 to 9 weeks *(22)*. Unfortunately, seed storage is not an ideal control measure; other research has indicated the presence of pathogens in seeds even after 38 weeks of storage at room temperature *(2, 37)*. Given the rapid increases in pathogen concentration routinely seen during the warm, wet sprouting process, even a single pathogen cell may result in contamination of a batch at very high concentrations.

Published research has revealed that none of the current disinfection treatments are able to completely eliminate pathogens on seeds, perhaps because sanitizers cannot reach bacteria trapped in cracks and crevices on the surface *(2, 15)*. Although in some cases a 5-log reduction may be achieved by presoaking seeds in sanitizers such as 20,000 ppm chlorine *(27)*, the log reduction of a particular pathogenic bacterium from this treatment is most likely to be far less *(26)*.

Irrigation water is easily collected for sampling and dilution and may provide a more representative picture of microbiological contamination status than would samples of unsprouted seeds or the sprouts themselves. For this reason, the U.S. Food and Drug Administration (FDA) has recommended that sprout producers test irrigation water for the presence of pathogens as a risk reduction measure *(27)*. Past research indicates that there is a strong correlation between microbial populations on alfalfa sprouts and populations in the irrigation water collected from those sprouts *(13)*, and some data suggest that irrigation water may contain microbial populations as high as 90% of those found in the alfalfa seeds or sprouts *(27)*.

Numerous studies have included investigations of mi-

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crobial growth dynamics during the sprouting process. Some experiments have been conducted using an overhead sprinkler irrigation system with small trays or bench-top sprouting chambers to mimic industrial conditions on a smaller scale *(9, 11, 19).* Other researchers have studied seeds irrigated in beakers or flasks *(10, 17, 20, 35, 39).* Although the bench-top method more closely mimics industrial conditions, the flask method is simpler and offers better biocontainment of pathogens in the laboratory. Few studies have included a systematic side-by-side comparison of different laboratory sprouting environments, although there is evidence that sprouting method has a minimal impact on total aerobic growth *(13).*

Evaluation of food safety research findings in actual food production environments has always been problematic because pathogens should not be brought knowingly into environments where food is produced. This problem has led to investigations into appropriate pathogen surrogates, i.e., organisms that have the same behavior as the pathogen of interest but are nonpathogenic. Examples of such surrogates are *Pediococcus* sp. NRRL B-2354, which has heat resistance similar to that of *Salmonella* Senftenberg 775W in fluid milk *(30), Listeria innocua* as a thermal inactivation surrogate for *Listeria monocytogenes (8),* and *Bacillus subtilis* as a UV surrogate for *Bacillus anthracis (28).* Research in our lab *(6)* and elsewhere *(40)* has included *Enterobacter aerogenes* B199A as a nonpathogenic surrogate for *Salmonella* in cross-contamination studies.

The main objective of this research was to address data gaps identified in a microbial risk assessment for *Salmonella* contamination and growth during the production of alfalfa sprouts *(25).* We assessed the effect of inoculum concentration and sprouting environment (bench-top chamber or flasks) on the growth of *Salmonella* Stanley and its potential surrogate *E. aerogenes* B199A. We also investigate the movement of bacteria from inoculated seeds to sprouts and irrigation water.

MATERIALS AND METHODS

Bacterial strain and growth media. *Salmonella* Stanley ATCC 7308 (Dr. Karl Matthews, Food Science Department, Rutgers University) was implicated in a 1995 outbreak *(31)* and transformed to constitutively express green fluorescent protein and to be resistant to ampicillin *(14). E. aerogenes* B199A, a nonpathogenic microorganism resistant to nalidixic acid *(40),* was investigated as a surrogate for *Salmonella* Stanley because it is easily enumerated and, like *Salmonella,* it is a gram-negative enteric bacterium.

Salmonella Stanley and *E. aerogenes* cells were grown for 18 h at 37°C with shaking (150 rpm) in tryptic soy broth (Difco, Becton Dickinson, Sparks, Md.) with 100 μg/ml ampicillin and 50 μg/ml nalidixic acid (Sigma, St. Louis, Mo.), respectively. Cells were harvested by centrifugation (Micro 12, Fisher Scientific, Pittsburgh, Pa.) at 5,000 $\times$ *g* for 5 min. The cell pellets were washed twice and resuspended in sterile phosphate-buffered saline (PBS; Fisher Scientific), and the mixture was adjusted by a spectrophotometer (Spectronic 501, Milton Roy, Ivyland, Pa.) to an optical density reading of approximately 0.5 at A660, corresponding to 10^8 CFU/ml. Appropriate 10-fold dilutions in PBS were made to give a final concentration of approximately 10^6 CFU/ml.

Inoculation and germination of alfalfa seeds. Dry alfalfa seeds (20 g; International Specialty Supply, Cookeville, Tenn.) were inoculated in a manner consistent with that reported previously *(15).* Seeds were soaked in 20 ml of pathogen suspension (10^6 CFU/ml) and mixed for 5 min. The suspension was decanted through cheesecloth, and seeds were placed under a laminar flow hood (Baker Company, Sanford, Maine) overnight to dry. Bacterial loads were determined 24 h before seeds were mixed with uninoculated seeds by homogenizing inoculated seeds with Butterfield's phosphate buffer in a stomacher (model 400, Seward, Worthington, UK) for 2 min.

To obtain the three desired inoculation concentrations (~3, ~30, and ~300 CFU/g), 5 to 50 g of inoculated seeds (10^6 CFU/g) and 40 to 180 g of uninoculated seeds were mixed gently and thoroughly in the appropriate ratios for 2 min in stomacher bags and then soaked for 4 h (0.45 kg of seeds in 2 liters of water). Soaked seeds were sprouted either on the bottom of plastic flasks or on screen trays in a bench-top sprouting chamber (Dr. Karl Matthews). The sprouting chamber was made of 6-mm-thick plastic, was completely enclosed and ventilated, and contained a spray irrigation system linked to a timer, drain system, and fluorescent lighting. Seeds were spread evenly over the bottom of the flasks or the chamber at the same industrial setting (0.45 kg/2,787 cm^2). The seeds were allowed to sprout at room temperature for 4 days, during which time they were either rinsed for 1 min every other hour from 10 a.m. to 10 p.m. each day in the flask or sprayed for 1 min in the chamber every other hour for 24 h each day with 200 ml of water per 929 cm^2. Spent irrigation water was collected for microbiological testing by decanting the flasks or emptying the bottom of the sprouting bed in the chamber. Sprouting experiments were repeated two to four times for each combination (inoculum concentration, sprouting environment, and bacterial strain) for a total of 17 independent experiments.

Quantification of bacteria during the sprouting process. One-gram seed samples were taken randomly from the sprouting environment and homogenized with 9 ml of Butterfield's phosphate buffer in a stomacher for 2 min. Water samples (1 ml) also were taken from irrigation water in the sprouting environment. A series of decimal dilutions of both the homogenates and the irrigation water were made, and 0.1 ml of each dilution was plated onto plate count agar (Difco, Becton Dickinson), tryptic soy agar (Fisher Scientific) containing 100 μg/ml ampicillin, and MacConkey agar (BBL, Becton Dickinson) containing 50 μg/ml nalidixic acid. Seed and sprout samples have a detection limit of 100 CFU/g (1 CFU/0.1 ml of 1-g sample plus 9 ml of buffer), and irrigation water has a detection limit of 10 CFU/ml (1 CFU/0.1 ml of irrigation water). After 24 h of incubation at 37°C, total plate counts were obtained from plate count agar, *E. aerogenes* was enumerated as purple colonies on the MacConkey's agar, and *Salmonella* Stanley was enumerated as green fluorescent colonies on tryptic soy agar.

Data analysis. Data were analyzed using the generalized linear model procedure from SAS version 8.2 (SAS Institute, Cary, N.C.). Microbial growth was modeled using the logistic equation:

$$y = \frac{k}{1 + \frac{k - n_0}{n_0} e^{-rt}}$$

where y is the log concentration of microorganisms at time t, n_0 is the initial log microbial concentration, e is the natural log base (~2.7182), r is the microbial growth rate, and k is the maximum log microbial concentration.

Statistical analysis of significant differences and calculation

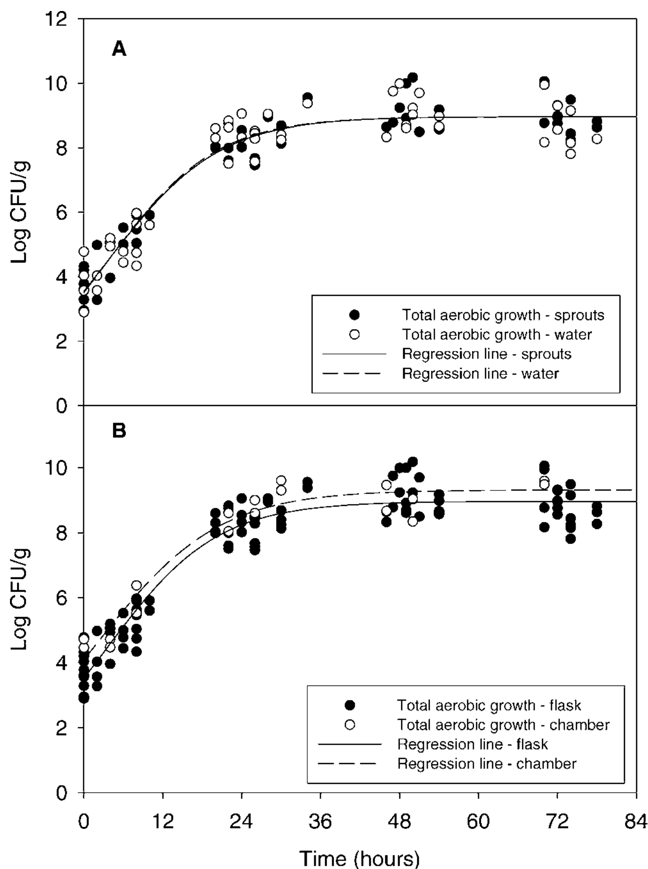

FIGURE 1. *Total aerobic bacterial growth during the sprouting process. (A) Total plate counts from seed and sprout samples (●) and irrigation water (○) were plotted at different time points during the sprouting process. Data were further analyzed by logistic regression; the solid line represents seed and sprout samples, and the broken line represents irrigation water samples. Data were collected from six sprouting experiments in flasks. (B) Data from seed and sprout samples or irrigation water samples were combined, and total aerobic counts from the sprouting chamber (○, broken line) or flask (●, solid line) were analyzed by logistic regression. The broken line represents chamber sprouting, and the solid line represents flask sprouting.*

of the microbial growth characteristics (growth rate r and maximum log concentration k) were generated by logistic regression using the SAS program.

RESULTS

Total plate count: sprouts versus water. Figure 1A presents a comparison of total aerobic plate count results from samples obtained from seeds and sprouts or from irrigation water from sprouting experiments done in flasks. The typical initial total plate count on seeds at time zero was 3.6 log CFU/g. With a generation time of about 3 h or a growth rate of about 0.1 log CFU/g/h, this initial population will increase to about 9 log CFU/g on sprouts after 24 to 48 h when the cells reach stationary phase. Total aerobic plate counts from the two environments (sprouts and water) were not significantly different at any point in the 4-day sprouting process; the logistic regression lines for the two data sets lie almost exactly on top of one another. Although results for seed and sprout samples were not different from those for water samples, total plate counts were variable and differed from the expected value (regression line) by ±1 log CFU/g at various time points (Fig. 1A).

TABLE 1. *Analysis of significant differences in bacterial growth rate and final concentrations as influenced by aspects of the experimental design*

Source of variability (experimental variable)	*P* value	
	Growth rates	Final concn
Apparatus type: flask or chamber	0.8705	0.7368
Bacteria type: total plate count, *Enterobacter aerogenes* count, or *Salmonella* Stanley count	0.5546	0.3863[a]
Inoculum concentration: ~3, ~30, or ~300 CFU/g or initial total plate count[b]	0.2684	<0.0001[c]
Sample type: sprouts or water	0.0164[c]	0.3136
Interaction		
Bacteria type × sample type	0.0801	0.1773
Bacteria type × apparatus type	0.0971	0.0869
Sample type × inoculum concentration	0.5385	0.5804
Sample type × apparatus concentration	0.6459	0.6652
Inoculum concentration × apparatus type	0.3977	0.4336

[a] Total plate count data were excluded from *P* value calculation for final concentrations because the confounding interaction with inoculum could not otherwise be eliminated.
[b] Initial total aerobic growth was estimated by logistic regression as 4,300 CFU/g.
[c] *P* value is significant (<0.05).

Total plate count: flask versus chamber. Figure 1B presents a comparison of total aerobic plate count results from samples incubated in flasks and those incubated in the chamber. Although the regression line modeling growth in the chamber lies slightly (~0.5 log CFU/g) above that for growth in flasks, further analysis revealed that neither the bacterial growth rates nor the final bacterial concentrations were significantly different between the flasks and the sprouting chamber (see results for apparatus type in Table 1) even though the two sprouting environments were different in scale (flasks as a small scale and chamber as a large scale) and irrigation system (rinse and decant versus overhead sprinkler).

***E. aerogenes* and *Salmonella* Stanley: sprouts versus water.** Further investigation of growth behavior of *E. aerogenes* and *Salmonella* Stanley during the spouting process produced results similar to those of the total aerobic growth experiments; neither growth rate nor final concentration of *E. aerogenes* or *Salmonella* Stanley was affected by sampling the sprouts or the water (Fig. 2). However, the concentrations of *E. aerogenes* and *Salmonella* Stanley (circles) were lower than the average total bacterial population (solid line) during the sprouting process. Statistical analysis also revealed that microbial growth rates and final bacterial

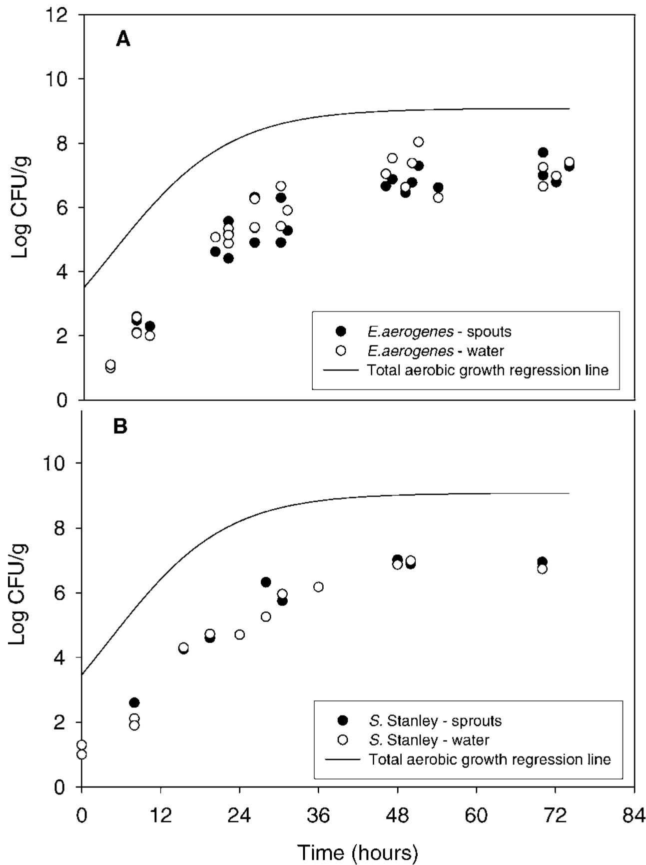

FIGURE 2. *Microbial growth in two types of samples at ~30 CFU/g: seed and sprout samples (●) or irrigation water samples (○). Solid line represents the logistic regression line from total aerobic growth. (A) Growth of* E. aerogenes *at ~30 CFU/g during the sprouting process. (B) Growth of* Salmonella *Stanley at ~30 CFU/g during the sprouting process.*

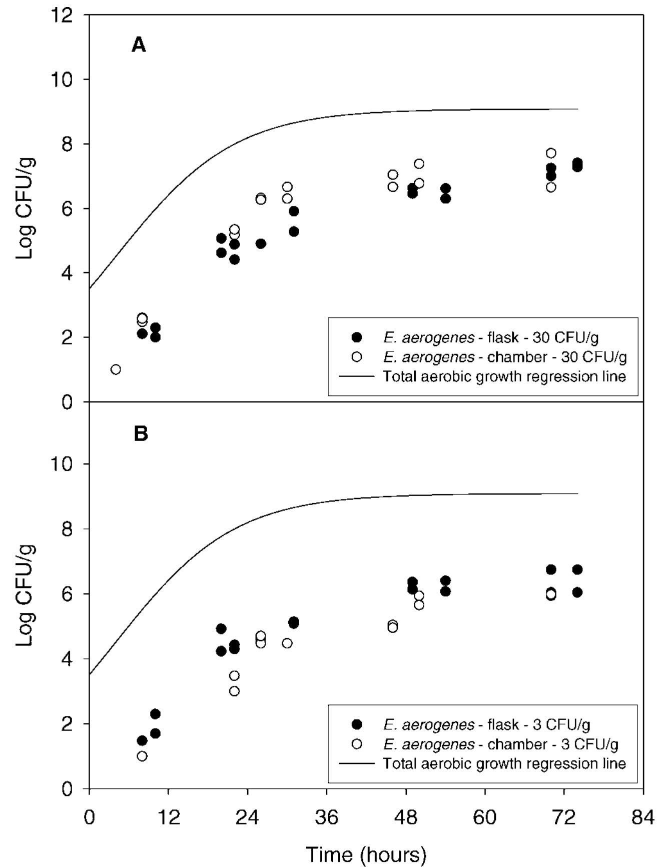

FIGURE 3. *Microbial growth in two types of sprouting environments: in flasks (●) or in a sprouting chamber (○). Solid line represents the logistic regression line from total aerobic growth. (A) Growth of* E. aerogenes *in two environments at ~30 CFU/g during the sprouting process. (B) Growth of* E. aerogenes *in two environments at 3 CFU/g during the sprouting process. Similar results were obtained at ~300 CFU/g.*

concentrations of *Salmonella* Stanley and *E. aerogenes* were not significantly different from one another but were significantly different (lower) from the total plate count data (see results for bacteria type and inoculum concentration in Table 1).

***E. aerogenes*: flask versus chamber.** Further investigation with *E. aerogenes* revealed that the sprouting environment (flask versus chamber) did not significantly affect recovery of the organism (Fig. 3), as was also seen with the total plate count results (Fig. 1B). This lack of difference was seen at initial inoculum concentrations of ~300 CFU/g (not shown), ~30 CFU/g (Fig. 3A), and ~3 CFU/g (Fig. 3B).

***E. aerogenes* versus *Salmonella* Stanley.** Figure 4 presents a comparison of the growth of *E. aerogenes* and *Salmonella* Stanley during the sprouting process after inoculation onto seeds at ~30 CFU/g (Fig. 4A) and ~3 CFU/g (Fig. 4B). Inspection of the data and the regression lines indicates that the growth of these two organisms was not significantly different. These findings are also supported by the results in Table 1 for bacteria type.

Inoculum concentration. The initial inoculum concentration of either *Salmonella* Stanley or *E. aerogenes* (~3, ~30, or ~300 CFU/g) influenced bacterial behavior during the sprouting process (Fig. 5). Final concentrations of *Salmonella* Stanley and *E. aerogenes* were proportional to starting concentrations, as indicated by the three dotted and dashed lines, respectively, in Figure 5. These dotted or dashed lines are also approximately parallel to the solid line representing the average amount of total aerobic bacterial growth during the sprouting process. Statistical analysis revealed that although bacterial growth rate was not influenced by initial concentration, final bacterial concentrations were strongly influenced ($P < 0.001$) by initial concentration (see results for inoculum concentration in Table 1).

Figure 5 also shows the influence of sample type (sprouts or water) on detection limit and hence time to detection of infection. The black arrow represents a detection limit of $\sim 10^2$ CFU/g in sprouting seeds, and the gray arrow represents a detection limit of ~10 CFU/ml in irrigation water. At an inoculum concentration of ~30 CFU/g, the presence of *E. aerogenes* or *Salmonella* Stanley was first detected within 4 h in irrigation water at ~1 log CFU/ml, 2 to 6 hours earlier than it was detected from seeds and sprouts at ~2 log CFU/g. Similar observations were obtained at inoculum concentrations of ~3 and ~300 CFU/g.

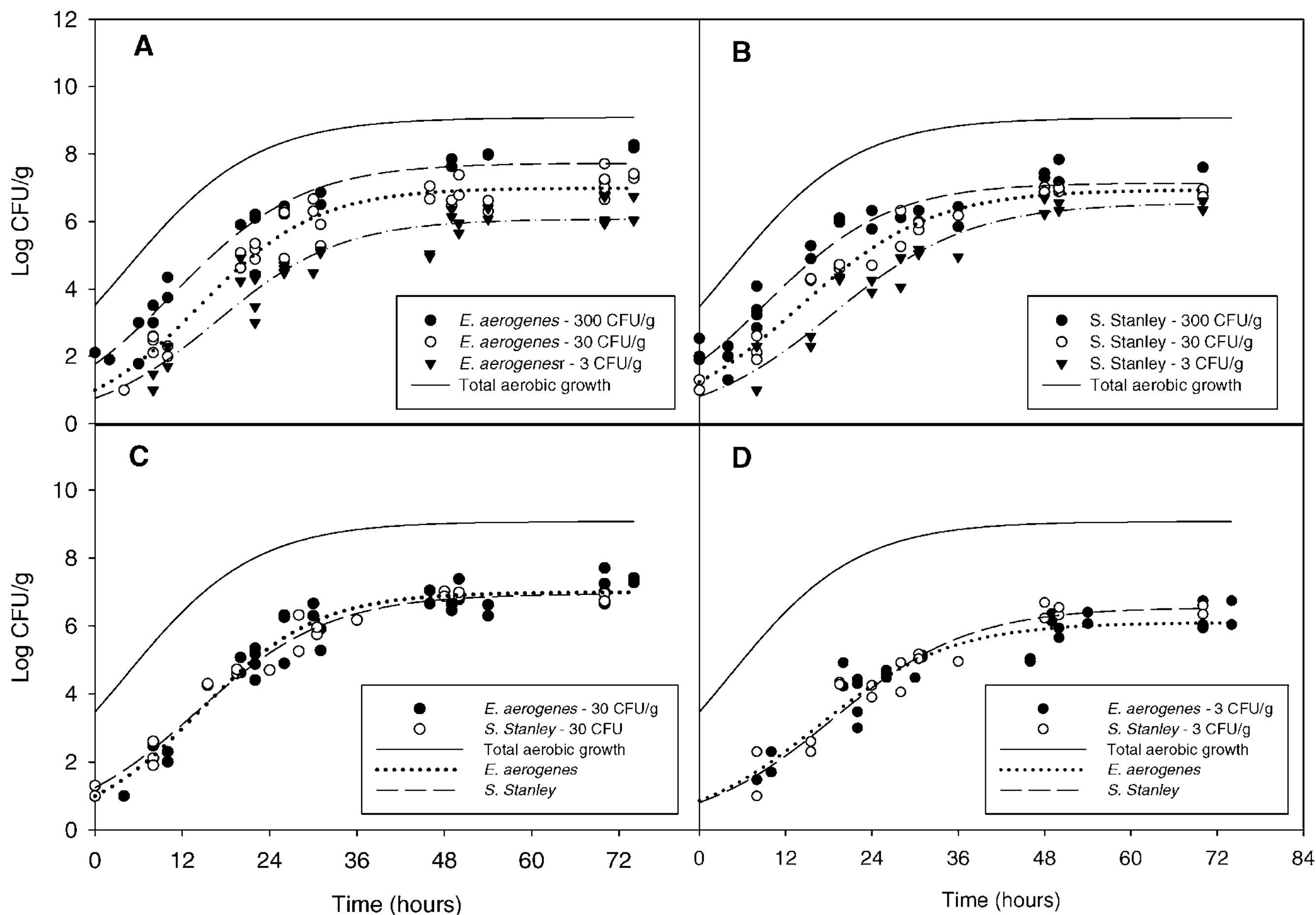

FIGURE 4. *Growth comparison between* E. aerogenes *and* Salmonella *Stanley. Data for growth during the sprouting process of* E. aerogenes *(A) or* Salmonella *Stanley (B) in different sample types and environment types were combined and plotted according to three inoculum concentrations: ~300 CFU/g (●), ~30 CFU/g (○), and ~3 CFU/g (▲). Data were further analyzed by logistic regression. Regression lines represent total aerobic growth (solid line) and initial inoculation concentrations of ~300 CFU/g (dashed line), ~30 CFU/g (dotted line), and ~3 CFU/g (dash-dot line). Comparison of growth of* E. aerogenes *(●) and* Salmonella *Stanley (○) also were made for initial inoculation concentrations of ~30 CFU/g (C) and ~3 CFU/g (D). Regression lines represent total aerobic growth (solid line),* E. aerogenes *growth (dotted line), and* Salmonella *Stanley growth (dashed line).*

Interaction of factors. In addition to the first-order effects (e.g., apparatus type and bacterial type), Table 1 includes the significance of interactions between factors. The *P* values indicate that no interactions between factors meet the standard criterion of significance ($P < 0.05$).

DISCUSSION

Much research had been conducted to investigate the various treatments used to eliminate pathogens from sprout seeds, including presoaking the seeds in 20,000 ppm chlorine, as recommended by the FDA *(27)*. Quantitative analysis of previously published studies indicates that the effects of such treatments are highly variable, even in the laboratory *(26)*. In naturally contaminated seed lots linked to confirmed outbreaks of foodborne illness, the contamination has usually been found to be approximately one pathogen cell per 100 g of seeds *(20)*. Very large (5 to 6 log CFU/g) increases in pathogen concentration during the sprouting process reported here and elsewhere *(1, 4, 5, 15, 19, 22, 34, 36)* means it is quite likely that even very low numbers of pathogens in seeds could multiply and reach levels likely to cause illness in some consumers. Given that decontamination alone may not be an effective risk mitigation strategy, irrigation water testing takes on increased importance.

In this study, there were no significant differences between sprout samples and irrigation water samples, which confirms previous findings that contamination levels in irrigation water are similar to those in the sprouts themselves *(13, 19, 34, 36)*. Although a prior analysis of published data conducted in our laboratory *(25)* revealed that there is a slightly higher average concentration (0.5 log CFU/g) in sprouts than in water, the range of possible differences reported in the literature clearly spans the region of no difference.

This research demonstrated that it is possible to do flask-based research that is suitably representative of research done in large bench-top sprouting chambers. In the final analysis, however, it may not be sufficient to confine research to bench-top sprouting chambers. Pilot plant or production scale experiments may still be required, and suitable nonpathogenic surrogates may be needed in these situations.

Water is essential to the sprouting process because it

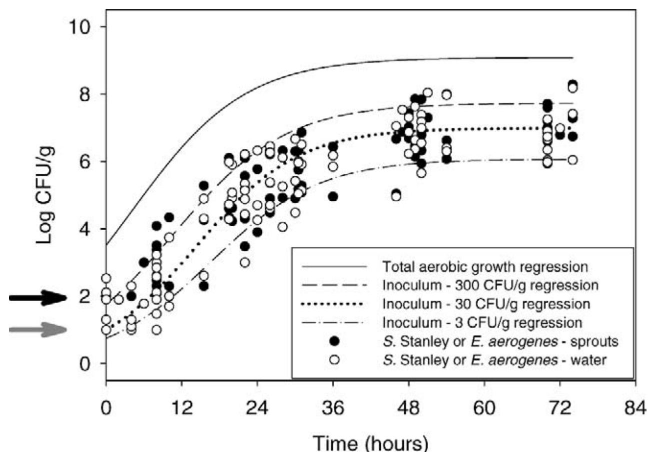

FIGURE 5. *Evaluation of samples of irrigation water allows early detection of microbial contamination. Microbial growth data from seeds and sprouts (●) and irrigation water (○) were plotted according to three inoculum concentrations. Growth curves represent total aerobic growth (solid line), ~300 CFU/g inoculum concentration (broken line), ~30 CFU/g inoculum concentration (dotted line), and ~3 CFU/g inoculum concentration (dash-dot line). Solid arrow represents detection limit for samples of seeds and sprouts (~10^2 CFU/g). Shaded arrow represents detection limit for samples of irrigation water (~10 CFU/ml). More data points were obtained for irrigation water samples in the first 8 h at all three inoculum concentrations.*

irrigates the sprouts, but water also aids pathogen migration. The practice of testing spent irrigation water can be used to monitor pathogen spread because it increases the overall probability of detecting contamination and aids in its early detection. At an inoculum concentration of only ~3 CFU/g, microbial populations reached 10 to 200 CFU/g in 8 h, at which point the contamination was detectable in irrigation water. A pathogen present at ~2 log CFU/g initially with a growth rate no faster than the average rate reported here (3.1-h doubling time) would require more than 27 to 36 h for concentrations to reach detectable levels. Thus, the common practice used by many sprout growers of collecting and sending irrigation water for microbiological testing after 48 h is likely to detect even low levels of contamination, assuming that such samples are representative *(18).*

The influence of sample type (sprouts or water) on detection limit also provides an explanation for the results for sample type shown in Table 1. Although sample type did not have a significant effect on final bacterial concentration ($P = 0.3136$), it did have a significant effect on growth rate ($P = 0.0164$) because of the limited number of growth rate data points for sprout and seed samples; many of these samples in the early part of the growth curve are below the detection limit. In contrast, water samples had lower detection limits (1 versus 2 log CFU/g) and hence provided more data points for the regression in the early part of the growth curve (see the preponderance of water samples [open circles] in the lower left corner of Fig. 5). Further data analysis (not shown) revealed that this trend was highly significant at the lowest inoculum concentration (~3 CFU/g), as can be seen by the difference in slope of the ~3 CFU/g regression line during the first ~8 h of the sprouting process.

The final concentrations of *E. aerogenes* and *Salmonella* Stanley during the sprouting process were proportional to their initial concentrations. This observation is consistent with a phenomenon known as the Jameson effect, i.e., two microorganisms inoculated together into medium or a food matrix will grow in numerical parity similar to their initial proportion until they reach the stationary phase, which is determined by the carrying capacity of the environment *(21).* The importance of this phenomenon in microbial risk assessment is now recognized *(32),* and the effect has been noted for a variety of pathogens *(3, 7, 16, 33).* This effect has been used recently in the control of pathogen growth in sprouts *(12).*

The existence of the Jameson effect also helps to explain some key differences between our study and that reported previously by Howard and Hutcheson *(19).* Because Howard and Hutcheson used sanitized seeds, their experiments started with a lower concentration of background microflora. That lower concentration meant that with a similar initial *Salmonella* concentration their experiments produced higher final *Salmonella* concentrations because *Salmonella* growth was not inhibited.

A nalidixic acid–resistant strain of *E. aerogenes* has been successfully used previously as a cross-contamination surrogate *(6, 24, 40).* The research presented here indicates that it also may be an appropriate surrogate for investigating the growth of *Salmonella* Stanley in the sprouting process at initial concentrations of ~3 to ~300 CFU/g.

The ability of *Salmonella* Stanley to grow rapidly to high concentrations during the sprouting process and the ability of inoculated organisms to spread easily from seed to sprout to irrigation water were confirmed in this study. Concentrations of *Salmonella* Stanley in irrigation water were not significantly different from those found on sprouts, also confirming the suitability of irrigation water samples for use in risk management studies. Final pathogen or surrogate concentrations were correlated with total bacterial concentrations in a manner consistent with the Jameson effect. We are the first researchers to show that the growth of *Salmonella* Stanley was not significantly different from that of the nalidixic acid–resistant *E. aerogenes,* a nonpathogenic organism that may be suitable for use as a surrogate in the study of growth dynamics in pilot laboratory experiments on a processing plant scale.

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